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Identification of Antigenic Regions in the GB Hepatitis Viruses GBV-A, GBV-B, and GBV-C

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The genomes of two novel members of the *Flaviviridae* associated with GB agent hepatitis (GB viruses A and B) were cloned and sequenced recently. The genome of a third novel virus (GB virus C), related to but distinct from GB viruses A and B, has also been identified and characterized. Overlapping clones encompassing the large open reading frames of these three viruses have been expressed in *E. coli* as CTP:CMP-3-deoxy-D-*manno*-octulosonate cytidylyltransferase (CKS) fusion proteins. Bacterial lysates were subjected to Western blot analyses using sera from GB agent-infected tamarins and human sera from various individuals with or "at risk" for non-A, non-B, non-C, non-D, non-E hepatitis. Antigenic regions were identified in the putative NS3, NS4, and NS5 proteins from all three viruses. An antigenic region was also identified in the putative core protein of GB virus B. Many of the clones identified originally as encoding antigenic proteins were quite large. To map these regions more narrowly, smaller overlapping clones were generated by polymerase chain reaction (PCR), expressed as recombinant CKS fusion proteins and tested by Western blot. Additionally, a λgt11 expression library was generated from infectious tamarin sera and immunoscreened. These studies have identified at least three epitopes in GB virus A, five epitopes in GB virus B and four epitopes in GB virus C.

KEY WORDS: non-A–E hepatitis, GB agent, PCR, *Flaviviridae*, CKS fusion

INTRODUCTION

Despite the introduction of reliable diagnostic assays for the detection of hepatitis C virus (HCV) and hepatitis E virus (HEV) [Dawson et al., 1992; Kuo et al., 1989; Schlauder and Mushahwar, 1994], there still remain cryptogenic cases of hepatitis of presumed viral origin [Alter, 1994; Peters et al., 1993; Thiers et al., 1993]. Recently, three new members of the *Flaviviridae* have been identified [Simons et al., 1995a,b] and the genomes of all three viruses have been cloned by polymerase chain reaction (PCR) walking techniques and sequenced [Leary et al., 1995b; Muerhoff et al., 1995; Simons et al., 1995b]. Representational difference analysis was used to isolate two of these viruses, GB virus A (GBV-A) and GB virus B (GBV-B), from GB hepatitis agent-infected primates. The third, GB virus C (GBV-C), was isolated from the serum of a GBV-B seropositive individual by reverse transcriptase (RT)-PCR using degenerate primers derived from GBV-A, GBV-B, and HCV sequences.

To explore the link further between these viruses and cases of hepatitis of unknown origin, it would be desirable to identify immunoreactive epitopes within the viral polyproteins in order to develop diagnostically useful reagents. To this end, regions spanning the large open reading frames (ORFs) of all three viruses were expressed in *E. coli* as fusions with CTP:CMP-3-deoxy-D-*manno*-octulosonate cytidylyltransferase (CKS) [Bolling and Mandecki, 1990]. To identify antigenic regions, the expressed proteins were examined by Western blot using sera from GBV-A and GBV-B infected primates and sera from humans with or "at risk" for non-A, non-B, non-C, non-D, non-E (non-A–E) hepatitis. These regions were defined further by evaluating the antigenicity of smaller expressed fragments generated by PCR. In addition, the construction and immunoscreening of a λgt11 expression library independently identified three of the regions encoded by GBV-B that had been found reactive by Western blot analysis. These methods have led to the identification of antigenic regions in GBV-A, GBV-B, and GBV-C which have been incorporated into serologic assays to identify individuals potentially infected with the GB viruses.

MATERIALS AND METHODS

Construction of Recombinant Plasmids

Plasmids pJO200, pJO201, and pJO202 each contain a modified *lac* promoter and a synthetic ribosome binding site linked to the *E. coli kdsB* gene, which encodes CKS. The plasmids are derived from pTB201 [Bolling

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and Mandecki, 1990] which has been modified to include a multiple cloning site for insertion of foreign genes at the 3′ end of the *kdsB* gene. The multiple cloning site is followed by translational stop signals in each frame and the *trpA* rho-independent transcriptional terminator. The three plasmids differ only in that each allows for insertion in a different reading frame within the multiple cloning site. GBV-A, GBV-B, GBV-C genomic fragments generated during the cloning of these viruses [Leary et al., 1995b; Simons et al., 1995a,b] were liberated from the pT7Blue T-vector (Novagen, Inc., Madison, WI) multiple cloning site and inserted into the multiple cloning sites of pJO200, pJO201, and pJO202 by standard methods [Sambrook et al., 1989]. Sequencing of the vector-insert junctions of each expression plasmid was carried out using the Sequenase version 2.0 kit (U.S. Biochemical Corp., Cleveland, OH).

Expression of Recombinant Proteins

E. coli XL1 Blue cultures (Stratagene Cloning Systems, La Jolla, CA) containing the CKS/GBV expression plasmids were grown at 37°C with shaking in media containing 32 g/L tryptone, 20 g/L yeast extract, 5 g/L NaCl, pH7.4, plus 100 mg/L ampicillin and 3 mM glucose. When the cultures reached an OD600 of between 1.0 and 2.0, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce expression. Growth was continued for an additional 3 hours at which time the cells were harvested by centrifugation.

Immunoblotting

E. coli cell pellets were resuspended in SDS/PAGE loading buffer (62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.1 mg/ml bromophenol blue) to an OD600 of 10 relative to the original OD600 of the culture, and boiled for 5 minutes. Aliquots were run on 10% SDS-polyacrylamide gels and either stained with Coomassie brilliant blue or transferred electrophoretically to nitrocellulose for immunoblotting as described by Towbin et al. [1979]. The blots were incubated in blocking solution (5% nonfat dry milk in Tris-buffered saline) for 30 minutes at room temperature followed by incubation for 1 hour at room temperature in either goat anti-CKS serum, pooled tamarin sera or human serum that had been preblocked against *E. coli* cell lysate [Sambrook et al., 1989]. The blots were washed with Tris-buffered saline (TBS), incubated for 1 hour at room temperature with horseradish peroxidase-conjugated rabbit anti-goat IgG or goat anti-human IgG as appropriate, then washed again. Bands were visualized in TBS containing 2 mg/ml 4-chloro-1-napthol, 0.02% hydrogen peroxide and 17% methanol.

Tamarin and Human Sera

A pool of GBV-convalescent sera from four tamarins diluted 1:200 in blocking solution was used for immunoblotting. The sera, mixed in equal proportion, were from T1034 (14 weeks post-GBV inoculation), T1048 (9 weeks post-GBV inoculation), T1051 (14 weeks post-GBV inoculation; 4 weeks post-GBV challenge) and T1057 (4 weeks post-GBV inoculation) [Schlauder et al., 1995a]. All human sera used for immunoblotting were diluted 1:100 in blocking solution. Human sera were as follows: GB: an aliquot obtained eight weeks postpresentation from the 34-year-old surgeon (with the initials GB) whose serum originally induced hepatitis in tamarins [Deinhardt et al., 1967]. WA-1: an individual from a West African population considered "at risk" for exposure to hepatitis viruses. This population has a high prevalence of exposure to HBV. Approximately 70% of the individuals are positive for anti-HBV core antigen and 14.1% of the individuals are HBV surface antigen-positive (indicating current HBV infection). In addition, 6.0% of the individuals from this population are anti-HCV-positive as determined by second generation anti-HCV EIA [Simons et al., 1995a]. WA-2: a different individual from the same West African population as WA-1. US-1: an intravenous drug user from the United States. This group has a high seroprevalence of antibody to HCV (99%) and to HBV (76%). US-2: an individual from the United States originally diagnosed with chronic active non-A–E hepatitis (subsequently found to be seropositive and PCR-positive for HCV and also found to be PCR-positive for GBV-C) [Leary et al., 1995a]. EA-1: an individual from East Africa diagnosed with acute non-A–E hepatitis.

Generation of Epitope Mapping Clones

GBV-A and -B PCR amplified genomic fragments were generated from T1053 cDNA [Simons et al., 1995b]. GBV-C PCR amplified genomic fragments utilized cDNA prepared from the serum of a GBV-C RNA-positive individual [Simons et al., 1995a]. Nucleic acid extractions and reverse transcription reactions were carried out as described previously [Simons et al., 1995b]. All PCRs utilized 1 μM primers for 35–40 cycles (94°C, 20–30 sec; 50–55°C, 30 sec; 72°C, 30–120 sec). Primers used to generate the clones each had a restriction site added at the 5′ end to facilitate cloning into the pJO201 multiple cloning site, preceded by six nucleotides to ensure complete digestion of the PCR product.

Generation of λgt11 Library

A pool of sera from three GBV-infected tamarins (T-1038, T-1049 and T-1055) [Schlauder et al., 1955a] was used to generate a virus stock as follows: approximately 3–4 ml of sera taken at the time the animals were autopsied from each of the three tamarins was pooled, providing a total volume of 11.3 ml. The pooled sera was clarified by centrifugation at 10,000 × g for 15 min at 15°C, and was then passed successively through 0.8, 0.45, 0.2, and 0.1 μm syringe filters. This filtered material was concentrated by centrifugation onto a 0.3 ml CsCl cushion (1.6 gm/ml density) in an SW41-Ti rotor at 41,000 rpm for 1 hour. The CsCl layer (approximately 0.6 ml) containing concentrated virus was aliquoted and stored at −70°C.

RNA was extracted from a 0.2 ml aliquot of the CsCl-concentrated virus and one-fourth of this was used for

cDNA synthesis. Phosphorylated, blunt-ended, double-stranded cDNA was prepared from the RNA using a commercially available kit (Stratagene, La Jolla, CA) and a double-stranded linker/primer [Reyes and Kim, 1991] was ligated to the cDNA ends. This provided all cDNAs in the mixture with identical 5′ and 3′ ends containing NotI and EcoRI restriction enzyme recognition sites. One-tenth of the cDNA was used in a 50 μl PCR containing the sense-strand primer at a final concentration of 1 μM (94°C, 30 sec; 55°C, 30 sec; 72°C, 1.5 min; 30 cycles). A 1 μl aliquot of the resulting products was reamplified as described above, digested with EcoRI, ligated to λgt11 vector DNA arms and packaged into phage heads. The resulting library contained approximately 1.73×10^6 phage at a recombination frequency of 89.3% with an average insert size of approximately 350 base pairs.

Immunoscreening of λgt11 Library

Two separate pools of tamarin sera were used for immunoscreening of the library. The first pool contained sera from two animals (T-1048 and T-1051). The T-1048 sera included aliquots from 9, 11, 13, 14, and 18 weeks postinoculation. The T-1051 sera included aliquots from 9, 11, 13, 14, 15, and 15.5 weeks postinoculation. The second pool contained aliquots from 6, 7, 9, 10, and 11 weeks postinoculation from a single animal (T1034). The procedure used for screening the library was based upon the method described by Young and Davis [1983] with modifications as described below. The primary antisera were preadsorbed against *E. coli* cell lysate prior to use. In the first experiment, 1.29×10^6 recombinant phage were immunoscreened with the T-1048/T-1051 antisera pool; in the second experiment 3×10^5 recombinant phage were immunoscreened with T-1034 antisera. In each experiment, the recombinant phage library was plated on a lawn of *E. coli* strain Y1090r- and grown at 37°C for 3.5 hours. The plates were overlaid with IPTG-saturated nylon filters and incubated at 42°C for 3.5 hours. The filters were then blocked in TBS containing 1% bovine serum albumin (BSA), 1% gelatin, and 3% Tween-20 (blocking buffer) for 1 hour at room temperature followed by incubation in primary antisera (1:100 dilution in blocking buffer) at 4°C for 16 hours. Primary antisera were removed and saved for subsequent rounds of plaque purification, and the filters were washed four times in TBS containing 0.1% Tween-20. The filters were incubated in blocking buffer containing ^{125}I-labeled goat anti-human IgG for 60 min at 22°C, washed as described above, and exposed to x-ray film.

RESULTS

Identification of GBV-B Immunoreactive Regions

The positions of the GBV-B expressed fragments along the GBV-B genome are shown in Figure 1. For each clone, the reading frame expressing full length protein was identified based on detection of a protein of the predicted size by anti-CKS Western blot, then confirmed further by DNA sequencing across the vector-insert junction. These proteins were examined for immunoreactivity with the tamarin serum pool by Western blot as described in Materials and Methods. As shown in Figure 2A, three of the CKS/GBV-B fusion proteins showed immunoreactivity. One of the reactive proteins, fragment B1, encompasses a segment of the putative core structural protein, while the other two are within the putative nonstructural regions NS3/4 (fragment B7) and NS5 (fragment B9). Fragments B7 and B9 are quite large, each encoding more than 500 amino acids of the GBV-B polyprotein.

In order to define more precisely the epitope(s) within the NS3/4 protein, a set of five smaller overlapping fragments (B12–B16) was generated by PCR (Fig. 1), expressed as CKS fusions and Western-blotted with tamarin sera as described above. Based on numbering as shown in Table I, the region of GBV-B encompassed by each of these fragments is indicated in Table II. The immunoreactivity of these fragments with the tamarin serum pool is shown in Figure 2B. Two immunoreactive regions were detected within the large NS3/4 protein (Fig. 1C), one in fragment B12 and one in fragment B14. Three additional fragments were generated to localize the immunoreactive regions further. Fragment B17, which showed no reactivity with the pooled tamarin serum, overlaps with fragment B12 by 49 amino acids at the N-terminus. Thus, the immunoreactive region within fragment B12 appears to lie in the C-terminal half of the fragment (residues 1256–1311). Fragments B18 and B19 overlap each other by 9 residues and encompass fragment B14. Both of these fragments were immunoreactive, B18 strongly and B19 weakly, indicating that there may be two epitopes within B14 or that the epitope may lie in the region of overlap between B18 and B19.

As was done for the NS3/4 region, a set of four smaller overlapping fragments (B20–B23) within the NS5 region was generated by PCR (Fig. 1C) and expressed as CKS fusions. The region of GBV-B encompassed by each of these is indicated in Table II. The immunoreactivity of these fragments with the tamarin sera is shown in Figure 2B. Two immunoreactive regions were localized within the large NS5 protein; one in fragment B21 and one in fragment B22. Two additional fragments were generated to map further the immunoreactive region within fragment B21. Fragments B24 and B25 overlap each other by 9 residues and encompass all but the first 9 residues of fragment B21. Fragment B24 was weakly reactive with the tamarin sera while fragment B25 was strongly reactive, suggesting that the immunoreactive region in B21 lies in the C-terminal half of the fragment.

Virus isolated from a pool of sera from three GBV-infected tamarins was used to generate a λgt11 library as described in Materials and Methods. This library was screened initially with the T1048/T1051 pooled sera. Two independent immunoreactive clones were found by this method (Fig. 1B). One is contained within the NS3 fragment B14 at residues 1423–1499, while the other is within the NS5 fragments B22 and B23 at residues 2192–2435. The library was subsequently screened in parallel with the T1048/T1051 and T1034 serum pools

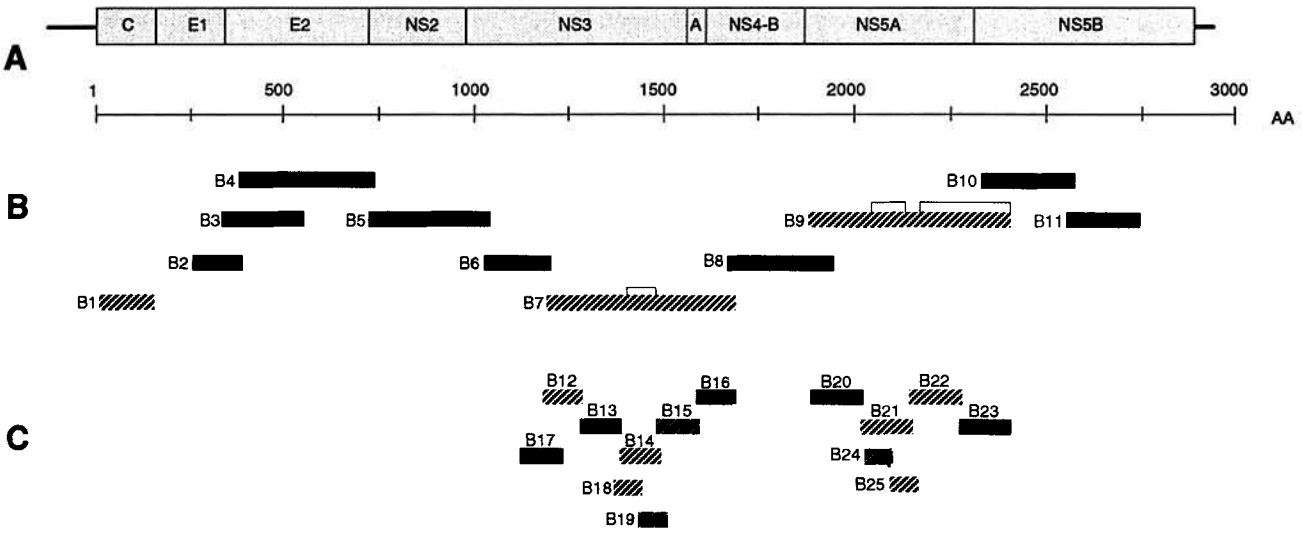

Fig. 1. Schematic representation of GBV-B genome and expressed proteins. **(A)** Putative genomic organization of GBV-B. **(B)** Fragments expressed from the GBV-B polyprotein. **(C)** Epitope mapping fragments within B7 and B9. Solid bars indicate non-reactive fragments, narrow cross-hatched bars represent reactive fragments, and wide cross-hatched bars represent weakly reactive fragments. The open bars above fragments B7 and B9 indicate the locations of the three immunoreactive regions found by immunoscreening of the λgt11 cDNA library.

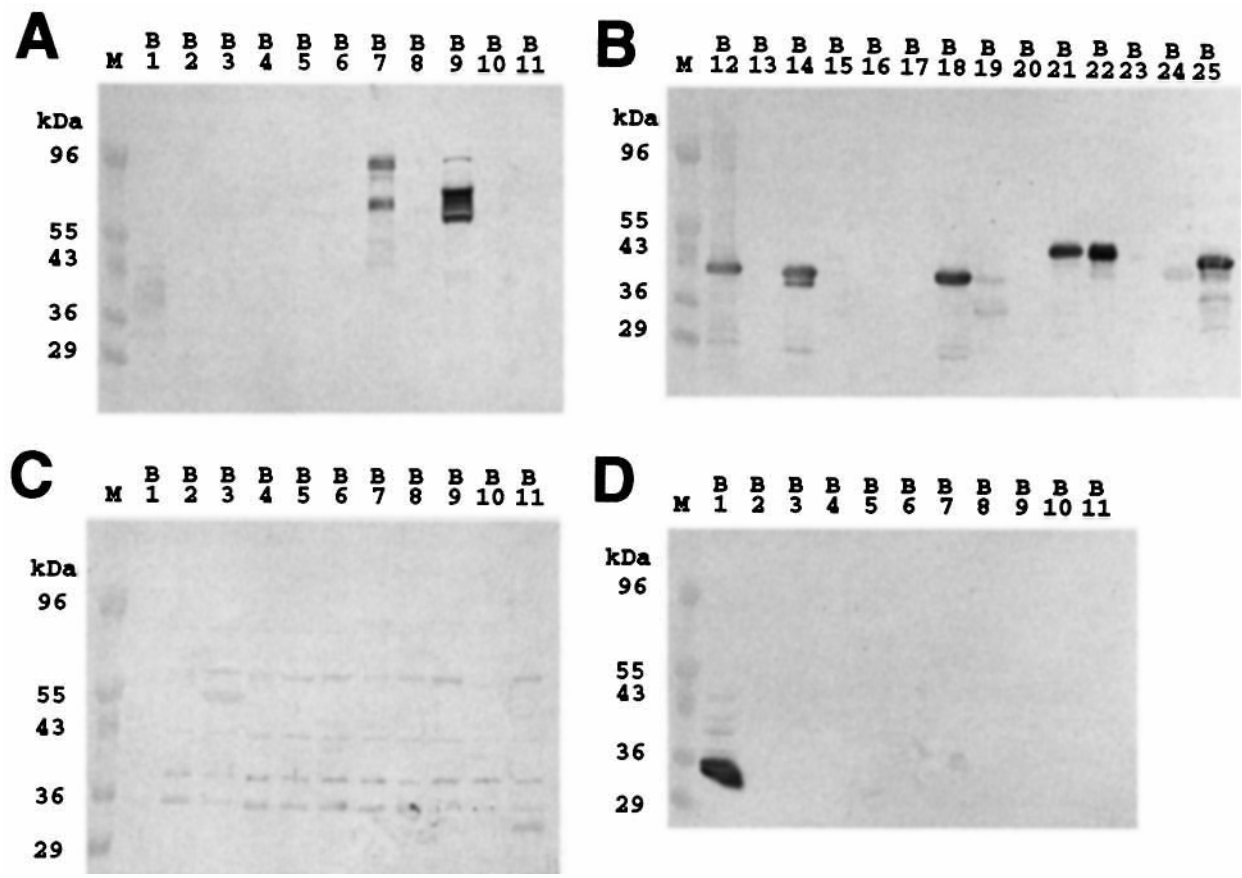

Fig. 2. Western blot analyses of expressed GBV-B proteins. *E. coli* lysates were separated by SDS-PAGE, transferred, then reacted with serum as described in the text. Lane designations refer to fragments from Fig. 1. **(A)** Reactivity of GBV-B fragments B1–B11 with pooled, GBV-infected tamarin sera. **(B)** Reactivity of GBV-B fragments B12–B25 with pooled, GBV-infected tamarin sera. **(C)** Reactivity of GBV-B fragments B1–B11 with human serum WA-1. **(D)** Reactivity of GBV-B fragments B1–B11 with human serum US#1. M, molecular weight markers (sizes as indicated).

TABLE I. Large Open Reading Frames (ORFs) Within GBV-A, GBV-B, and GBV-C

Virus	Nucleotide position of ORF[a]	Total no. of amino acids in ORF	No. of amino acids beginning with first Met[b]
GBV-A	540–9458	2,972	2,962
GBV-B	443–9040	2,865	2,864
GBV-C	344–9064	2,906	2,874

[a]From GenBank accession nos. U22303 (GBV-A), U22304 (GBV-B) and U36380 (GBV-C).
[b]As previously described (14), the GBV-B ORF including the probable initiator methionine has been identified. For neither GBV-A nor GBV-C is it clear whether the first identified methionine in the ORF is the true initiator (11, 14). Therefore, numbering used to identify antigenic regions from GBV-B is based on starting from the first methionine in the ORF, while for GBV-A and GBV-C, the numbering starts from the first amino acid residue in the ORF.

TABLE II. Epitope Mapping Fragments

Clone designation[a]	Virus	Region amplified[b]	
		Nucleotide	Amino acid[c]
A14	GBV-A	6123–6467	1862–1976
A15	GBV-A	6438–6782	1967–2081
A16	GBV-A	6753–7097	2072–2186
A17	GBV-A	7068–7412	2177–2291
A18	GBV-A	7383–7727	2282–2396
A19	GBV-A	7698–8045	2387–2502
B12	GBV-B	4064–4378	1207–1311
B13	GBV-B	4355–4681	1304–1412
B14	GBV-B	4658–4987	1405–1514
B15	GBV-B	4964–5293	1507–1616
B16	GBV-B	5270–5581	1609–1712
B17	GBV-B	3869–4210	1142–1255
B18	GBV-B	4616–4840	1391–1465
B19	GBV-B	4814–5038	1457–1531
B20	GBV-B	6158–6568	1905–2041
B21	GBV-B	6542–6949	2033–2168
B22	GBV-B	6923–7333	2160–2296
B23	GBV-B	7307–7747	2288–2434
B24	GBV-B	6569–6793	2042–2116
B25	GBV-B	6767–6988	2108–2181
C15	GBV-C	2939–3268	866–975
C16	GBV-C	3239–3592	966–1083
C17	GBV-C	3563–3916	1074–1191
C18	GBV-C	3887–4240	1182–1299
C19	GBV-C	4211–4564	1290–1407
C20	GBV-C	4535–4882	1398–1513
C21	GBV-C	4853–5212	1504–1623
C22	GBV-C	5183–5509	1614–1722
C23	GBV-C	5480–5860	1713–1839
C24	GBV-C	5831–6190	1830–1949
C25	GBV-C	6161–6511	1940–2056
C26	GBV-C	6482–6835	2047–2164
C27	GBV-C	6806–7156	2155–2271
C28	GBV-C	7127–7471	2262–2376

[a]Refer to Figures 1, 3, or 5.
[b]Region from GBV-A (Genbank accession no. U22303), GBV-B (Genbank accession no. U22304) or GBV-C (Genbank accession no. U36380) viral sequence amplified.
[c]Refer to Table I.

and a third clone was identified which was reactive with both serum pools. It is located within the NS5 fragment B21 at residues 2065–2157 (Fig. 1B). Identification of epitopes in NS3/4 and NS5 by two independent methods (Western blotting of CKS fusion proteins and λgt11 immunoscreening) as well as the identification of the immunoreactive putative core protein by Western blotting supports the notion that these regions contain the immunodominant linear epitopes which elicit an immune response in tamarins infected with GBV-B.

The CKS/GBV-B proteins were examined by Western blotting with a number of human sera from individuals with or "at risk" for non-A–E hepatitis. The blot shown in Figure 2C demonstrates the immunoreactivity of these proteins with serum from WA-1. The NS3/4 fragment (B7) that was reactive with the tamarin sera also exhibited immunoreactivity with this human serum sample. This serum was also reactive with an additional fragment (B3) from the region encoding a portion of the putative E2 structural protein. Another human serum from an intravenous drug user (US-1) was immunoreactive with fragment B1 that encompasses the putative core protein (Fig. 2D). Additional human sera from individuals diagnosed with acute non-A–E hepatitis also demonstrated immunoreactivity with the core, NS3/4 and/or NS5 regions identified as antigenic with tamarin sera (data not shown). GB serum (from the human source of the "GB hepatitis agent") was utilized in Western blots against the GBV-B fragments. None of the GBV-B proteins exhibited immunoreactivity with this serum (data not shown).

Identification of GBV-A Immunoreactive Regions

The locations of the expressed fragments within the GBV-A genome are shown in Figure 3. The correct reading frame for each clone was identified as described for GBV-B (above). These proteins were examined by Western blot analysis with the tamarin serum pool as described for GBV-B. None of the GBV-A fragments had reactivity with the tamarin serum pool, despite the fact that GBV-A was previously shown by PCR to be present in the serum of at least one of these tamarins after inoculation [Schlauder et al., 1995a]. Western blot analyses using sera from single bleed dates of GBV-A PCR-positive tamarins were undertaken to determine whether some of the tamarins mounted a weak response to GBV-A proteins. GBV-A immunoreactivity was not detected using sera from the GBV-A infected tamarins. This is consistent with the lack of detectable seroconversion in these GBV-A infected tamarins as detected by ELISAs that utilized CKS fusion proteins from GBV-A NS3, NS4 and NS5 (fragments A5, A6, A9, A10 and A11 from Fig. 4B) [Schlauder et al., 1995a].

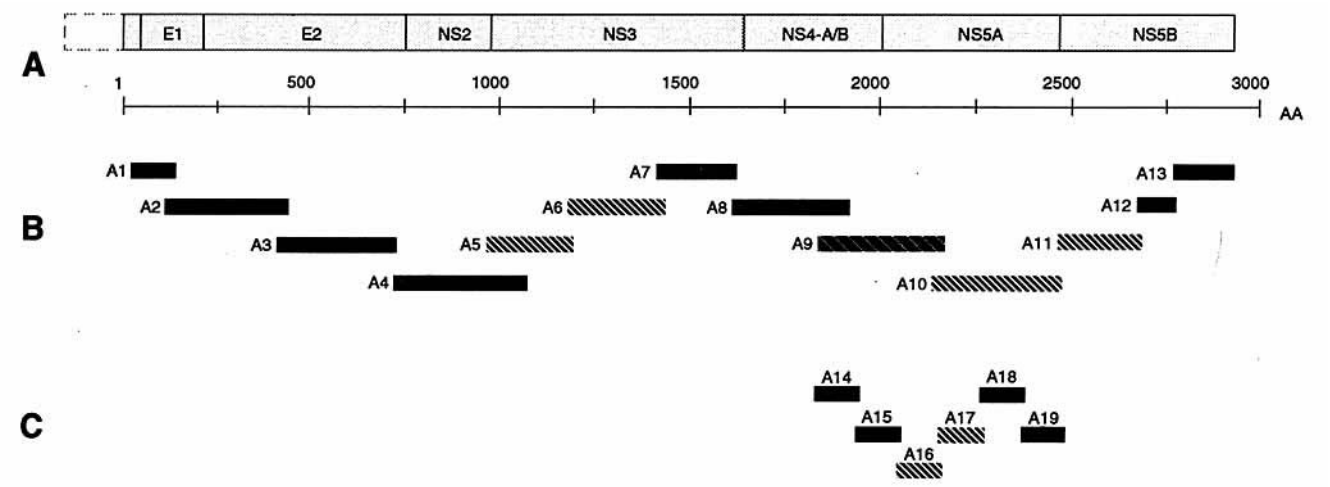

Fig. 3. Schematic representation of GBV-A genome and expressed proteins. **(A)** Putative genomic organization of GBV-A. **(B)** Fragments expressed from the GBV-A polyprotein. **(C)** Epitope mapping fragments within A9 and A10. Solid bars indicate non-reactive fragments, narrow cross-hatched bars represent reactive fragments, and wide cross-hatched bars represent weakly reactive fragments.

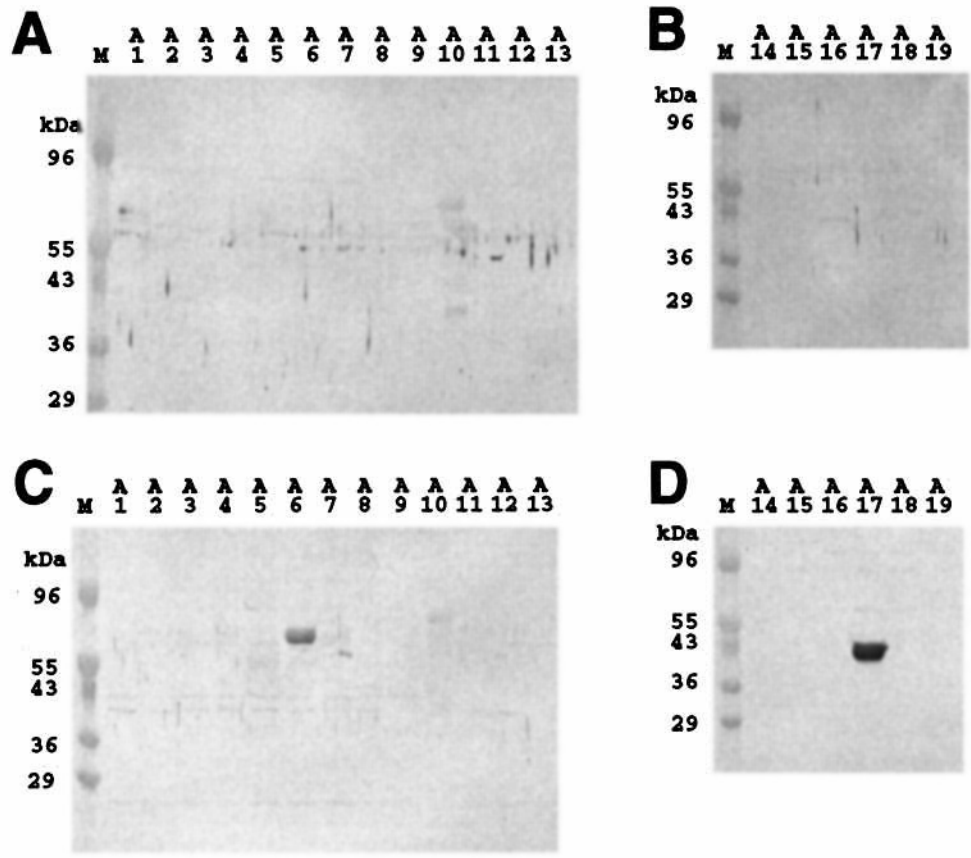

Fig. 4. Western blot analyses of expressed GBV-A proteins. *E. coli* lysates were separated by SDS-PAGE, transferred, then reacted with serum as described in the text. Lane designations refer to fragments from Fig. 3. **(A)** Reactivity of GBV-A fragments A1–A13 with GB serum. **(B)** Reactivity of GBV-A fragments A14–A19 with GB serum. **(C)** Reactivity of GBV-A fragments A1–A13 with human serum EA-1. **(D)** Reactivity of GBV-A fragments A14–B19 with human serum EA-1. M, molecular weight markers (sizes as indicated).

In contrast to the apparent lack of immunoreactivity of the GB-infected tamarin sera with GBV-A encoded proteins, GB serum was immunoreactive with two overlapping fragments in NS4/5A (Fig. 4A). Fragment A9 was weakly reactive while fragment A10 was more strongly reactive. A set of six overlapping fragments was generated by PCR to further localize the epitope(s) within these two fragments (Fig. 3). Based on numbering as in Table I, the region of GBV-A encompassed by each of these fragments is indicated in Table II. Figure 4B shows the reactivity of these six fragments with the GB serum. Two fragments that overlap each other by ten amino acids were reactive, one (A16) more strongly than the other (A17), while four of the fragments (A14, A15, A18, and A19) did not exhibit immunoreactivity. It is not clear whether the reactivity observed with GB serum against fragments A16 and A17 is due to a single shared epitope or whether the reactivity reflects the presence of more than one epitope. It would appear, however, to localize the strongest reactivity with the GB serum to the 27 amino acid region of GBV-A held in common between fragments A10 and A16 (residues 2160–2186).

Additional human sera from individuals with or "at risk" for non-A–E hepatitis were examined for immunoreactivity against the GBV-A proteins by Western blotting. Results with one of these sera (EA-1), from an East African patient with acute non-A–E hepatitis, are shown in Figure 4C. This serum exhibited immunoreactivity with the A10 fragment previously shown to be reactive with the GB serum, as well as with two additional overlapping fragments in NS3; i.e., A5 and A6. The fragment A9/A10 epitope mapping clones were examined with this serum (Fig. 4D) and fragment A17 was positive. Since no reactivity was observed with fragment A16, it is not clear whether this serum is recognizing the same epitope as that identified by the GB serum. Additional sera from individuals with or "at risk" for non-A–E hepatitis were also found to be reactive with fragments A5, A6, and/or A10. Several sera also identified fragment A11 as reactive (data not shown).

Identification of GBV-C Immunoreactive Regions

The identification of seroreactive regions from GBV-A and GBV-B has led to the development of ELISAs using these proteins (Dawson et al., manuscript in preparation). Sera from individuals with or "at risk" for non-A–E hepatitis identified as reactive in one or more of these assays were tested by PCR for the presence of GBV-A or -B as previously described. A number of samples were found to contain sequences from a third virus, GBV-C [Simons et al., 1995a]. This virus has been molecularly cloned [Leary et al., 1995b] and cDNA fragments were expressed as CKS fusions.

The locations of the expressed fragments within the GBV-C genome are shown in Figure 5. The correct reading frame for each clone was identified as was done for the GBV-A and -B clones. These proteins were examined by Western blot analyses for immunoreactivity with the tamarin serum pool and with the GB serum as described above. None of the GBV-C fragments were reactive with either of these sera (data not shown). Human sera from individuals with or "risk" for non-A–E hepatitis were examined by Western blotting for immunoreactivity with the GBV-C proteins. One of these was from the West African population considered "at risk" for exposure to hepatitis viruses described above, but was from a different patient (designated WA-2). The other was from a North American individual (designated US-2) originally diagnosed with chronic active non-A–E hepatitis who was found subsequently to be HCV RT-PCR-positive. This individual was GBV-C RT-PCR-positive as well. The reactivity of the GBV-C proteins with these sera is shown in Figure 6A and C. Fragments C6, C7 and C9–C12 were reactive with one or the other of these sera. Fragment C8 was found to be weakly reactive with an additional serum from a multiply transfused individual considered at risk for non-A–E hepatitis (data not shown). The reactive fragments C6–C12 span NS3 through a portion of NS5B.

A set of fourteen overlapping PCR fragments was generated to further localize the epitope(s) in fragments C6 through C11 (Fig. 5C). Based on numbering as in Table I, the region of GBV-C encompassed by each of these fragments is indicated in Table II. The Western blots in Figure 6B and D show the reactivity of these fragments with human sera WA-2 and US-2, respectively. Fragments C17, C22, C26, C27, and C28 exhibited immunoreactivity with at least one of the sera. Fragment C17 localizes the C6 reactive region to residues 1074–1191 and fragment C22 localizes the C9 reactive region to residues 1614–1722. Fragments C26, C27 and C28, spanning residues 2047–2376, were all reactive, indicating the presence of at least two epitopes in this region. Although fragment C7 had shown reactivity previously with serum WA-2, the epitope mapping fragments in this region did not show reactivity. It is possible that the new fragments split an epitope such that it is not fully represented in any of these proteins. Alternatively, the reactivity seen with the larger protein may result from a conformational epitope not fully represented in the smaller products. An additional anomaly observed was that although the M47 serum was not reactive with the protein encoded by fragment C11, the smaller mapping fragment C27, which is completely within C11, was reactive with this serum. It is possible that this is due to the presence of a conformationally dependent epitope.

DISCUSSION

Each of the three GB viruses, GBV-A, -B and -C, contains a large ORF encoding a polyprotein of greater than 2,800 amino acids. Since these viruses have only recently been isolated and characterized molecularly, there is no existing information regarding the locations of antigenically important regions. By expressing overlapping clones encompassing the GBV-A, -B and -C putative polyproteins in *E. coli* as fusions with CKS, followed by Western blot analyses, we have ascertained the locations of seroreactive regions in each of these viruses.

For GBV-B, which appears to cause acute resolving

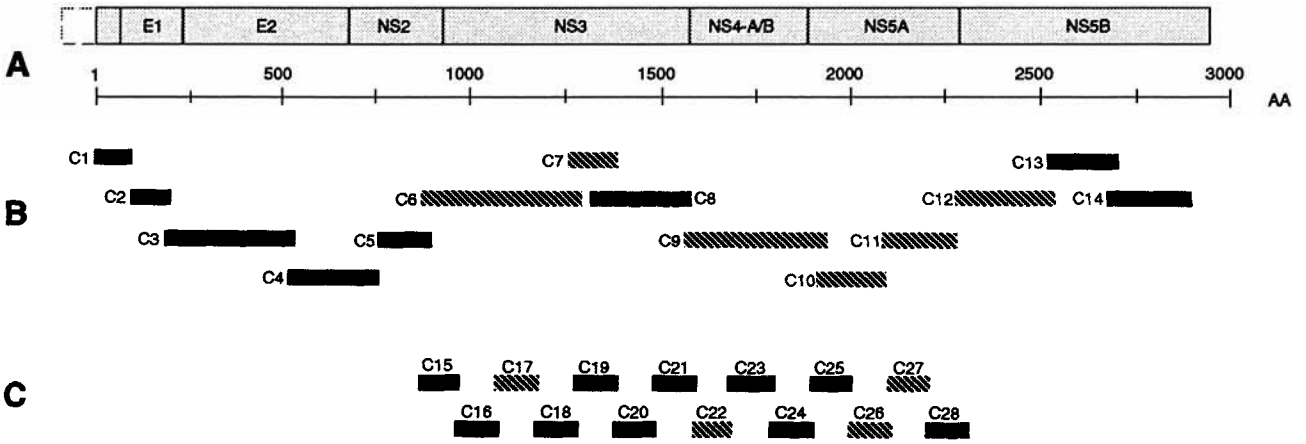

Fig. 5. Schematic representation of GBV-C genome and expressed proteins. **(A)** Putative genomic organization of GBV-C. **(B)** Fragments expressed from the GBV-C polyprotein. **(C)** Epitope mapping fragments within C6–C11. Solid bars indicate non-reactive fragments, narrow cross-hatched bars represent reactive fragments, and wide cross-hatched bars represent weakly reactive fragments.

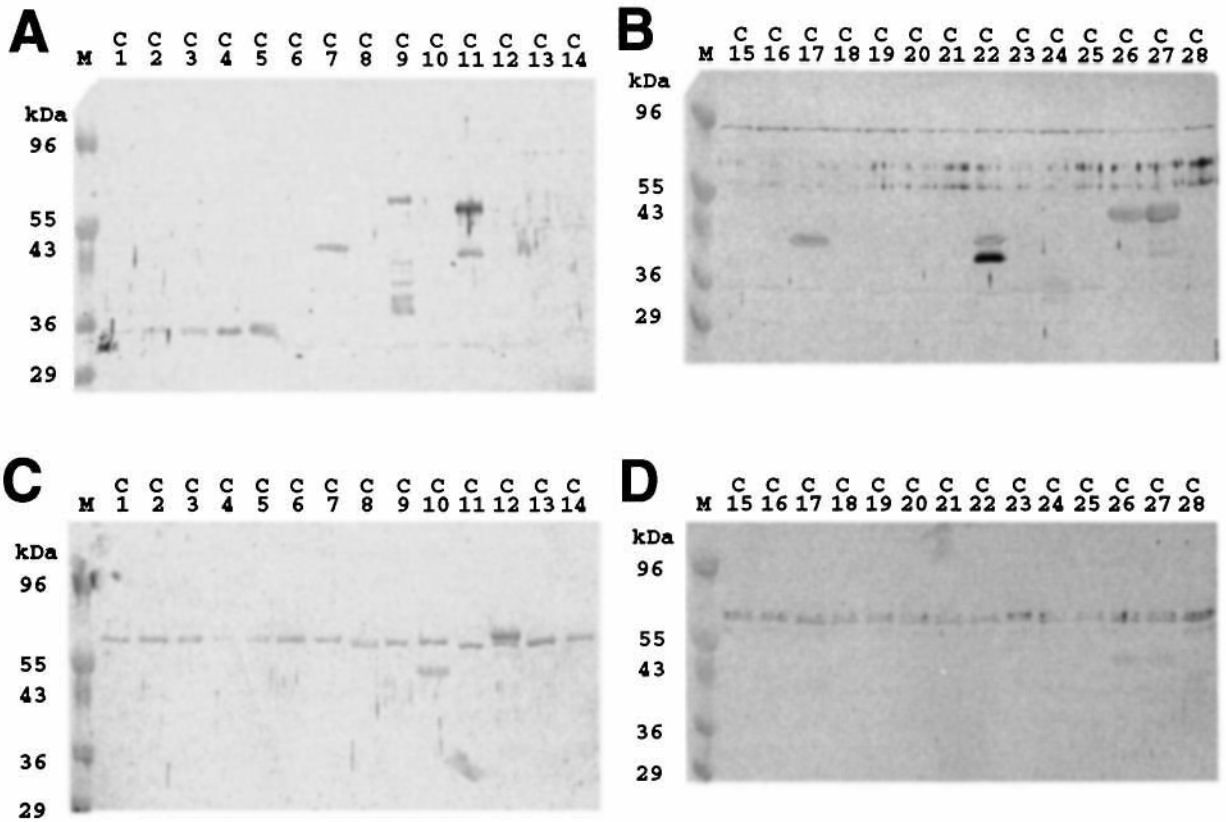

Fig. 6. Western blot analyses of expressed GBV-C proteins. *E. coli* lysates were separated by SDS-PAGE, transferred, then reacted with serum as described in the text. Lane designations refer to fragments from Fig. 5. **(A)** Reactivity of GBV-C fragments C1–C14 with human serum WA-2. **(B)** Reactivity of GBV-C fragments C15–C28 with human serum WA-2. **(C)** Reactivity of GBV-C fragments C1–C14 with human serum US-2. **(D)** Reactivity of GBV-C fragments C14–C28 with human serum US-2. M, molecular weight markers (sizes as indicated).

hepatitis in tamarins [Schlauder et al., 1995a], the antigenic regions (located in the putative core, NS3/4 and NS5 proteins) were identified using pooled sera from GBV-B infected animals. Expression of small overlapping portions of the NS3/4 and NS5 proteins localized at least two immunoreactive regions in each. Immunoscreening of a λgt11 expression library confirmed the locations of one of the NS3/4 regions and two of the NS5 regions. The core, NS3/4 and NS5 immunoreactive regions map to residues 3–118, 1256–1311 and 1422–1499, and 2065–2157 and 2192–2296, respectively.

When Western blot analyses with various human sera from individuals with or "at risk" for non-A–E hepatitis were carried out, each of the three large regions of GBV-B identified as immunoreactive with tamarin sera was also found to be immunoreactive with some human sera. An additional region within the putative structural glycoprotein E2 was also immunoreactive with the US-1 serum. We did not find individual human sera that were immunoractive with all of the regions identified by the tamarin sera. This is consistent with the immunoreactivity found by ELISAs using these proteins when serum from individual tamarins was used for the analysis rather than a pool of sera from several animals [Schlauder et al., 1995a]. Although all of the animals with GB hepatitis had detectable immunoreactivity with at least one of the three proteins, and each of the three proteins was immunoreactive with sera from at least one animal, no single animal was strongly immunoreactive with all three proteins. In addition, the immunoreactivity seen with human sera is somewhat weaker than that seen with the tamarin sera. This may reflect a difference in antibody titer in GBV-B infected tamarins versus GBV-B infected humans. Alternatively, this may be the result of significant sequence differences between the GBV-B isolated from tamarins and the human viral sequence which in turn would affect the sequences of the viral protein epitopes. The GBV-B virus inoculated into the tamarins used for these studies was derived from material which had been passaged serially through tamarins 11 times. It is also possible that GBV-B is a tamarin virus and the immunoreactivity we have detected in humans is due to cross-reactivity. Until GBV-B is isolated from a human source, this issue will remain unresolved.

No reactivity was detected against the GBV-B proteins using eight week postpresentation GB serum (from the human originator of the "GB hepatitis agent"). This does not eliminate the possibility that GB was infected with GBV-B. In tamarins infected with this virus, the immune response to GBV-B proteins appears to be of short duration, in some cases lasting only a few weeks [Schlauder et al., 1995a]. It may be that GB had little or no immune response to the epitopes examined, or that the response was transient. Conformational epitopes from regions such as the envelope glycoproteins E1 or E2 expressed in mammalian cells may be necessary to detect a longer duration immune response to this virus in both tamarins and humans.

Two overlapping immunoreactive regions were identified within NS5 when the eight week postpresentation GB serum was used to examine the GBV-A proteins. This reactivity was quite weak as detected by Western blot. The weakness of the reactivity could be due to differences in the viral sequences resulting from passage through tamarins as discussed above for GBV-B. It may also be a result of the age and storage conditions of the GB serum used for detection. This serum had been stored at −20°C for over 30 years. Alternatively, GBV-A may be a tamarin virus, as indicated by the presence of GBV-A-related sequences in uninoculated animals [Schlauder et al., 1995b]. The reactivity of GB serum may therefore be due to cross-reactivity or nonspecific reaction with the GBV-A proteins. As mentioned above, tamarins known to be infected with GBV-A do not mount a detectable immune response to the GBV-A proteins as determined by Western blot or ELISA. We have been unable to determine whether GB serum contained GBV-A or -B by RT-PCR [Schlauder et al., 1995a]. This may be due to the fact that proteins such as antibodies have a longer half-life in serum than nucleic acids, particularly RNA.

We have found a number of human serum samples from individuals with or "at risk" for non-A–E hepatitis, in addition to GB, that exhibit immunoreactivity with GBV-A fragment A10. Epitope mapping of the GBV-A NS5 region using GB and other human sera indicated that there may be more than one epitope in the A9–A10 region. This reactivity maps to the region encoded by fragments A16 and A17 (GBV-A residues 2072–2291). Reactivity was also observed in the NS2/3 region of GBV-A with several of the human sera, but no mapping has been done to identify the locations of the reactive epitopes.

Sera from individuals with or "at risk" for non-A–E hepatitis, including one that was GBV-C RT-PCR-positive, were used to map the locations of seroreactive regions encoded by the GBV-C large ORF. Proteins encoded by a large portion of the nonstructural region of GBV-C were found to be immunoreactive, although all sera were not reactive with the same regions. Epitope mapping identified specific regions within NS3, NS4 and NS5 (residues 1074–1191, 1614–1722, and 2047–2376, respectively) as reactive. The NS5 region was identified using both a GBV-C RT-PCR-negative serum (WA-2) as well as GBV-C RT-PCR-positive serum (US-2). Interestingly, the US-2 serum was only reactive with a protein from this region when it was expressed as a smaller epitope mapping fragment of 117 amino acid residues fused to CKS (fragment C27), but not when a larger region of 202 amino acid residues (fragment C11) which completely encompassed the smaller fragment, was expressed. This same phenomenon was observed with additional human sera (data not shown). Recognition of this epitope may be dependent upon the surrounding sequence in which it is presented.

Immunoreactive regions have been identified in the putative nonstructural proteins, particularly NS3, NS4 and NS5, from all three GB viruses, as well as in the putative core protein of GBV-B. A similar pattern of immunoreactivity has been seen in individuals infected with HCV, which is the closet relative of the GB viruses [Leary et al., 1995b; Muerhoff et al., 1995]. The HCV

core protein and the nonstructural regions NS3, NS4 and NS5 contain a large concentration of immunodominant epitopes identified with both recombinant proteins and synthetic peptides [Chien et al., 1992; Khudyakov et al., 1995]. Serum from individuals infected with HCV is often immunoreactive with epitopes from several of these regions [Chien et al., 1992; Khudyakov et al., 1995; Lok et al., 1993; Yatsuhashi et al., 1992]. In addition, somewhat more distant relatives of the GB viruses such as the pestivirus bovine viral diarrhea virus and the flavivirus Japanese encephalitis virus contain one or more immunodominant epitopes in their structural proteins as well as in their respective NS3 proteins [Collett et al., 1988; Donis and Dubovi, 1987; Mason et al., 1987]. No immunoreactivity has been reported, however, in the other nonstructural proteins of these flavi- and pestiviruses. Thus, some correlation is seen between the regions of the GB viruses identified in our studies by Western blot analyses and regions identified as being immunoreactive in HCV and other members of the *Flaviviridae*. ELISAs have been developed using these identified regions from all three of the GB viruses, and studies are currently underway on human sera using these assays as well as RT-PCR to determine the clinical relevance of these newly identified viruses.

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